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Paroxysmal Nocturnal Hemoglobinuria: Analysis of the Effects of Mutant *PIG-A* on Gene Expression

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Compelling evidence indicates that mutations in *PIG-A* are necessary for the development of paroxysmal nocturnal hemaglobinuria (PNH), however, it is unclear why mutant *PIG-A* stem cells have a selective advantage. Further, multiple, discrete *PIG-A* mutations have been detected in the peripheral blood and bone marrow of patients with PNH, but the contribution of the different mutant clones to hematopoiesis is variable. This observation implies that factors in addition to mutant *PIG-A* influence the proliferative properties of the abnormal cells. To investigate the etiology of the selective advantage and the clonal dominance in PNH, gene expression in cells with mutant *PIG-A* was analyzed. Representational difference analysis was used to compare the pattern of cDNA expression between a human lymphoblastoid cell line with mutant *PIG-A* and its wild-type counterpart. These experiments demonstrated that the pattern of gene expression was different between the two cells lines in that the *PIG-A* mutant cells failed to express antiquitin mRNA. Transfection of the mutant cells with normal *PIG-A* restored expression of glycosyl phosphatidylinositol anchored proteins but not antiquitin. These experiments demonstrate that differences in the pattern of gene expression can occur independent of the *PIG-A* mutation. Depending upon the functional properties of the involved genes, these differences could influence the proliferative properties of *PIG-A* mutant cells and contribute to the selective advantage and clonal dominance that characterize PNH. Am. J. Hematol. 61:221–231, 1999. © 1999 Wiley-Liss, Inc.

Key words: paroxysmal nocturnal hemoglobinuria; *PIG-A;* glycosyl phosphtidylinositol; hemolytic anemia

INTRODUCTION

Somatic mutations of the X-linked gene *PIG-A* that arise in a primitive hematopoietic stem cell provide a clear explanation for the hemolytic anemia that is a characteristic clinical manifestation of paroxysmal nocturnal hemoglobinuria (PNH). *PIG-A* encodes a protein that is essential for the normal synthesis of the glycosyl phosphatidylinositol moiety that serves as a membrane anchor for a functionally diverse group of proteins [1]. Among these glycosyl phosphatidylinositol anchored proteins (GPI-AP) are two important regulators of the complement system—decay-accelerating factor (DAF, CD55) [2] and membrane inhibitor of reactive lysis (MIRL, CD59) [3]. Erythrocytes that are the progeny of stem cells with mutant *PIG-A* are abnormally susceptible to complement-mediated lysis because they are deficient in DAF and MIRL [4]. Patients with PNH experience chronic and acute episodes of intravascular hemolysis because their erythrocytes lack these regulatory factors that normally protect the cell from complement-induced injury [5].

The mechanism by which mutations in *PIG-A* account for the hemolytic component of PNH is obvious, and that *PIG-A* mutations are necessary for the development of PNH is incontrovertible. However, the process by which the mutant stem cells expand such that the clinical manifestations of the disease become apparent is largely speculative. In vivo experiments using homologous recombination suggest that the *PIG-A* mutation per se does

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not endow affected cells with a proliferative advantage [6] but other observations indicate that the hematopoietic stem cells from patients with PNH do have a relative growth or survival advantage [7,8]. Together, these findings are consistent with the hypothesis that *PIG-A* mutations are necessary, but not sufficient, for the production of PNH that is clinically apparent. Strong support for the concept that PNH arises as a consequence of a specific pathophysiological process affecting bone marrow elements is provided by studies that have identified as many as four separate *PIG-A* mutations in the hematopoietic cells of a single patient [9,10]. These remarkable results suggest a powerful selection pressure that favors emergence of *PIG-A* mutant cells [11]. In cases where multiple *PIG-A* mutant cells have been identified, not all of the affected cells contribute equally to hematopoiesis [9,10].

PNH may also be seen in association with other clonal myelopathies [5,12] suggesting that in some instances the *PIG-A* mutation acts in concert with mutations affecting other genes to enhance the proliferative properties of the affected clone. In this case, the *PIG-A* mutation may be part of a multistep process analogous to that by which malignant neoplasms develop through clonal evolution. Approximately one third of untransplanted patients with aplastic anemia develop PNH [13–15], suggesting that PNH frequently develops on a background of bone marrow injury, immunosuppression, or both. In addition to PNH, patients with aplastic anemia are at risk for developing other clonal myelopathies including myelodysplastic disease and acute leukemia [16]. Thus, an element of genetic instability coupled with altered immune surveillance may contribute to the process by which PNH develops in association with aplastic anemia. Together, these observations suggest that factors in addition to the *PIG-A* mutation contribute to the pathophysiological process that gives rise to PNH.

The growth and survival characteristics of cells are determined by the pattern of gene expression, and both genetic and epigenetic factors influence these processes. To gain insights into the molecular basis of the selective advantage and clonal dominance associated with PNH, studies designed to determine whether mutant *PIG-A* influences the pattern of gene expression were undertaken. Representational difference analysis (RDA) was used to compare amplifiable restriction fragments from cDNA prepared from a *PIG-A* mutant cell line with those of its wild-type counterpart. These experiments suggest that genetic or epigenetic events that occur independent of mutant *PIG-A* may contribute to the pathophysiology of PNH.

MATERIALS AND METHODS

Cells

A human B lymphoblastoid cell line (JY) deficient in GPI-AP (JY-PI$^-$) and its wild-type counterpart (JY-WT) were provided by Dr. Gregory Spear (Rush-Presbyterian-St. Luke's Medical Center, Chicago, IL). The cells were maintained in culture using RPMI supplemented with 10% fetal calf serum (FCS). The two cell lines cells were originally developed by Hollander et al. [17] by treating JY cells with the mutagen ethylmethanesulfonate and subsequently selecting for GPI-AP negative cells by using anti-DAF antibody and fluorescence-activated cell sorting. The GPI-AP deficient cell-line used in the present experiments was designated JY-33 by Hollander et al. [17] while the wild-type line was designated JY-25 [17]. Published methods were used to generate antigen nonspecific cultured T cells from a normal volunteer [18].

Flow Cytometry

DAF (CD55) and MIRL (CD59) expression by JY-WT and JY-PI$^-$ cells was analyzed by flow cytometry according to the method of Endo et al. [9]. Monoclonal anti-DAF (clone 1H4) was a gift from Dr. Wendell Rosse (Duke University Medical Center, Durham, NC) and monoclonal anti-CD59 (clone 1F5) was a gift of Dr. Hidechika Okada (Nagoya City University, Nagoya, Japan). Ten thousand gated events per sample were analyzed by using a FACScan (Becton Dickinson, Research Triangle Park, NC) provided by Huntsman Cancer Institute Flow Cytometry Facility of the University of Utah.

Northern Analysis

The method for characterization of PIG-A mRNA expression has been published previously [9]. By using the same procedure, antiquitin, DAF [19], and MIRL [20,21] specific probes were used to analyze total or poly (A+) RNA [22] from JY-WT and JY-PI$^-$ cells. Published methods were used for radiolabeling of the oligonucleotide probes [9]. After preparation of the autoradiography, the radioactivity was removed by placing the blots in a container and adding 500 ml of a boiling solution of 0.25% sodium dodecylsulfate SDS, 0.1× SSC. The container was vigorously shaken using orbital rotation until the solution had cooled (~15 min). Next, the solution was poured out of the container and the procedure was repeated once. Subsequently, the amount and integrity of the RNA contained in each lane of the blot was assessed by analysis with a radiolabeled glyceraldehyde phosphate dehydrogenase (GAPDH) probe (a gift from Dr. Graydon Harker, VA Medical Center, Salt Lake City, UT).

Sequencing of *PIG-A*

Direct nucleotide sequencing of PIG-A using genomic DNA from JY-PI$^-$ cells was performed by using methods that have been published previously [8,9,23].

RDA

The method was developed originally by Lisitsyn and colleagues [24,25] for analysis of genomic DNA. To

compare patterns of gene expression, the procedure was modified by Hubank and Schatz [26]. In the present study, minor modifications of that method were used. The Copy Kit cDNA Synthesis System from Invitrogen (San Diego, CA) was used to prepare oligo dT primed, double-stranded cDNA from poly(A+) RNA isolated from JY-WT and JY-PI$^-$ cells. Aliquots of 10 μg of double-stranded cDNA were incubated at 37°C with 100 units of *Bgl*II (New England Biolabs, Beverly, MA). After 4 hr, the samples were washed twice in 2 ml of 10 mM Tris-HCl, pH 8.0, containing 1 mM ethylenediaminetetraacetic (EDTA) using a Centricon 100 concentrator (Amicon, Beverly, MA). The oligonucleotide adaptor/primers were prepared by the DNA/Peptide Core Facility of the University of Utah Health Sciences Center. Each set of adaptor/primer was desalted by washing three times with 2 ml of deionized water by using a Centricon 3 concentrator (Amicon). After generation of the amplicons, the samples were digested with *Bgl*II and subsequently washed three times with 2 ml of TE using a Centricon 100 concentrator. Following digestion of single-stranded cDNA with mung bean nuclease (New England Biolabs), the samples were washed once with 2 ml of TE using a Centricon 100 concentrator. The volume was adjusted to 200 μl with TE, and difference products were generated by PCR as described in the protocol [26]. Prior to ligation of the appropriate adaptor primer, the difference products were digested with *Bgl*II and subsequently washed twice in 2 ml of TE by using a Centricon 100 concentrator.

Cloning of Difference Products

After three rounds of hybridization subtraction, difference product 3 (DP-3) was directly cloned by using the Original TA Cloning Kit (Invitrogen). Plasmid DNA was isolated by using the Qiaprep Spin Plasmid Kit (Qiagen, Chatsworth, CA). Following excision using EcoRI (New England Biolabs), the size of each cloned insert was estimated by agarose gel electrophoresis and ethidium bromide staining. Nucleotide sequencing of the inserts was performed by the University of Utah Health Sciences Center Sequencing Facility. Sequence similarity searches were conducted by using MacVector 4.5.3 software (Eastman Kodak Company Scientific Imaging Systems, New Haven, CT). Insert sequence was compared with the Basic Local Alignment Search Tool Nucleotide (BLASTN) database.

Southern Analysis

Aliquots of cDNA from JY-WT and JY-PI$^-$ representational amplicons were electrophoresed in 1% agarose gels. Southern analysis was performed by using standard methods [9]. The blot was analyzed by using the RDA generated antiquitin specific probe. The procedure for radiolabeling of the probe has been published previously [9].

Transfection of JY-PI$^-$ Cells

After washing in RPMI medium, JY-PI$^-$ cells were resuspended to 2.5×10^7/ml in RPMI containing 10% fetal calf serum. Two aliquots of 400 μl of cells were transferred into 0.4 cm width electroporation cuvettes (Gibco BRL Life Technologies, Grand Island, NY). To one aliquot of cells was added 20 μg of pEBPIG-A vector and to the other aliquot was added 20 μg of pEBCAT vector. The vectors were a gift from Dr. Taroh Kinoshita (Osaka University, Osaka, Japan). The pEBPIG-A is an Epstein-Barr virus-based expression vector that contains a 3.6 kb full-length *PIG-A* cDNA and a hygromycin resistance gene [27,28]. The pEBCAT vector is the same except it contains the chloramphenicol acetyltransferase (CAT) gene. The samples were electroporated at 240 volts and 1180 μF [29]. The cells were resuspended in 5 ml of RPMI containing 10% fetal calf serum and cultured at 37°C in a 5% CO_2 incubator. After 24 hr, 5 ml of RPMI containing 10% fetal calf serum and 400 μg/ml of Hygromycin B (Calbiochem-Novabiochem, San Diego, CA) were added. The cells were maintained in culture in medium containing 200 μg/ml of Hygromycin B and at timed intervals, aliquots of the cells were analyzed for expression of GPI-AP by flow cytometry and for expression of CAT by ELISA (Boehringer Mannheim, Indianapolis, IN).

Antiquitin Reverse Transcriptase-polymerase Chain Reaction (RT-PCR)

Total RNA isolated from normal T cells, JY-WT, JY-PI$^-$, JY-PI$^-$ transfected with pEBPIG-A, and JY-PI$^-$ transfected with pEBCAT was used as template to synthesize oligo dT primed first-strand cDNA by using the cDNA Cycle Kit (Invitrogen). Aliquots were used to amplify antiquitin cDNA by using the following primers: sense, 5′-CAAGCTGCCTGGTGCAATTTG-3′; antisense, 5′-TCCATAACCTTGCCCCCATAG-3′ or actin cDNA by using the following primers (gifts from Dr. Mark Holguin, VA Medical Center, Salt Lake City, UT): sense, 5′-GGCACCACACCTTCTACAATG-3′; antisense, 5′-TAGCACAGCCTGGATAGCAAC-3′. The PCR reaction mixture consisted of 2 μl of sample cDNA, 5 μl of 10× buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 25 mM $MgCl_2$; 0.01% gelatin), 1 μl of dNTP mixture (25 mM each), 1 μl of sense primer (200 ng/μl), 1 μl of antisense primer (200 ng/μl), 1 μl of Taq polymerase (1.5 U/μl), and 39 μl of deionized water. Thirty cycles were performed using the following conditions: denature for 1 min at 94°C; anneal for 2 min at 55°C; extend for 3 min at 72°C; final extension for 7 min at 72°C. Aliquots of 10 μl of each sample were analyzed by agarose gel electrophoresis and ethidium bromide staining. The antiquitin PCR product is 486 bp and the actin PCR product is 162 bp.

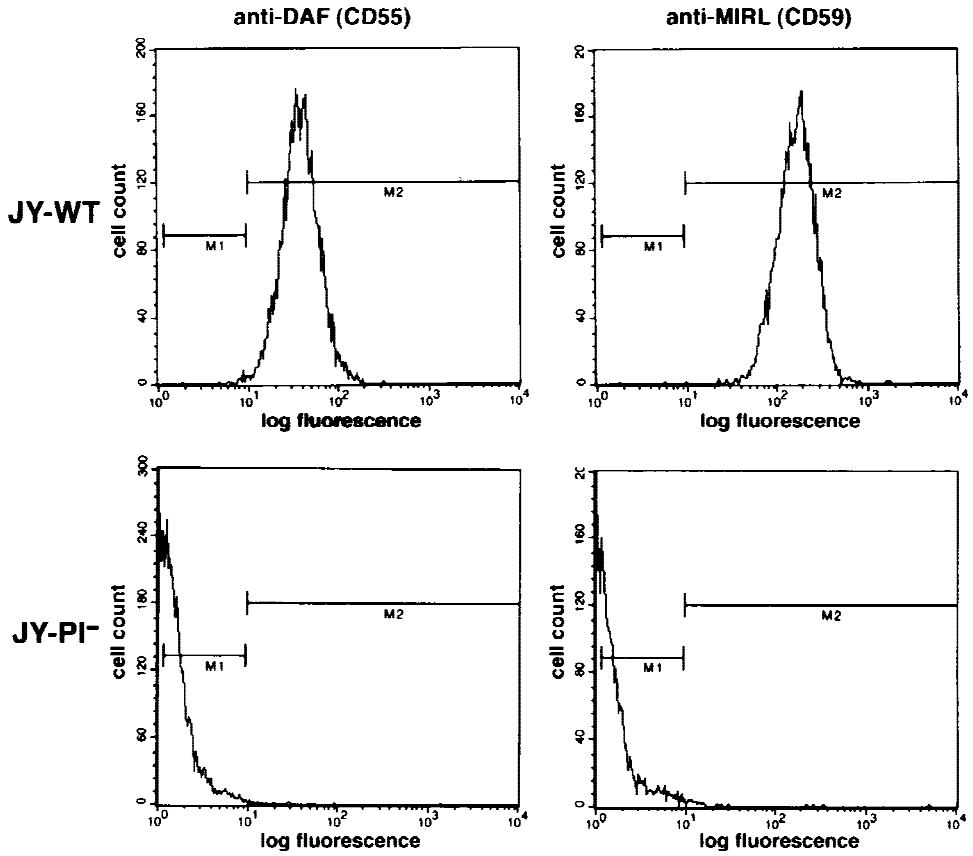

Fig. 1. Analysis of expression of GPI-AP by JY-WT and JY-PI⁻ cells. Expression of two GPI-AP was analyzed by flow cytometry by using monoclonal anti-DAF (CD55) or anti-MIRL (CD59) as primary antibodies. JY-PI⁻ cells were deficient in both DAF and MIRL.

RESULTS

Characterization of JY-PI⁻ Cells

Analysis using flow cytometry confirmed that the JY-PI⁻ cells did not express GPI-AP (Fig. 1). Northern analysis demonstrated that expression of PIG-A mRNA was abnormally low suggesting that a mutation in PIG-A accounted for failure of the JY-PI⁻ cells to express GPI-AP (Fig. 2). This interpretation was confirmed by genomic sequencing of PIG-A that demonstrated a G to A transition involving the 3′ splice site of intron 4 [the wild-type aa**g**GTT was changed to aa**a**GTT (intronic sequence shown in lower case letters and exonic sequence shown in upper case letters, with the mutated site shown in bold type)]. By altering the splice site, this mutation produced a frameshift deletion that introduced a premature stop signal at codon 329 (the open reading frame of normal *PIG-A* consists of 484 codons). These experiments confirm that the abnormal expression of GPI-AP by JY-PI⁻ is a consequence of mutant PIG-A.

Comparison of the Pattern of Gene Expression between JY-WT and JY-PI⁻ Cells by Using RDA

Conceptually, RDA is similar to other subtractive methods in that one DNA population (the driver) is hybridized in excess against a second population (the tester) to remove common sequences, thereby enriching for sequences unique to the tester. RDA was used in the present experiments because by combining subtractive hybridization with both representation and kinetic enrichment the degree of purification of sequences unique to the tester is markedly enhanced [24–26].

After preparation of double stranded cDNA from JY-WT and JY-PI⁻ cells, representational amplicons were generated by digesting the samples with *Bgl*II, ligating the samples with a set of adaptor/primers, and amplifying the restriction fragments by using PCR. Analysis by agarose gel electrophoresis and ethidium bromide staining showed a series of bands of varying intensity over a size range of approximately 1,500 to 150 bp (Fig. 3A). There was no obvious difference in the JY-WT amplicon compared to the JY-PI⁻ amplicon.

To generate the first difference product (DP-1), the representational amplicons were digested with *Bgl*II to remove the adaptor/primers, and after washing, a portion of each sample was ligated with a different set of adaptor/primers in order to create the respective testers. Two sets of subtraction/hybridizations were performed in parallel. In set A, the JY-WT representation amplicon was used as the driver and the JY-PI⁻ representational amplicon was used as the tester. In set B, the subtraction/hybridization was performed in the opposite direction (i.e., the JY-PI⁻ amplicon served as the driver, and JY-WT amplicon

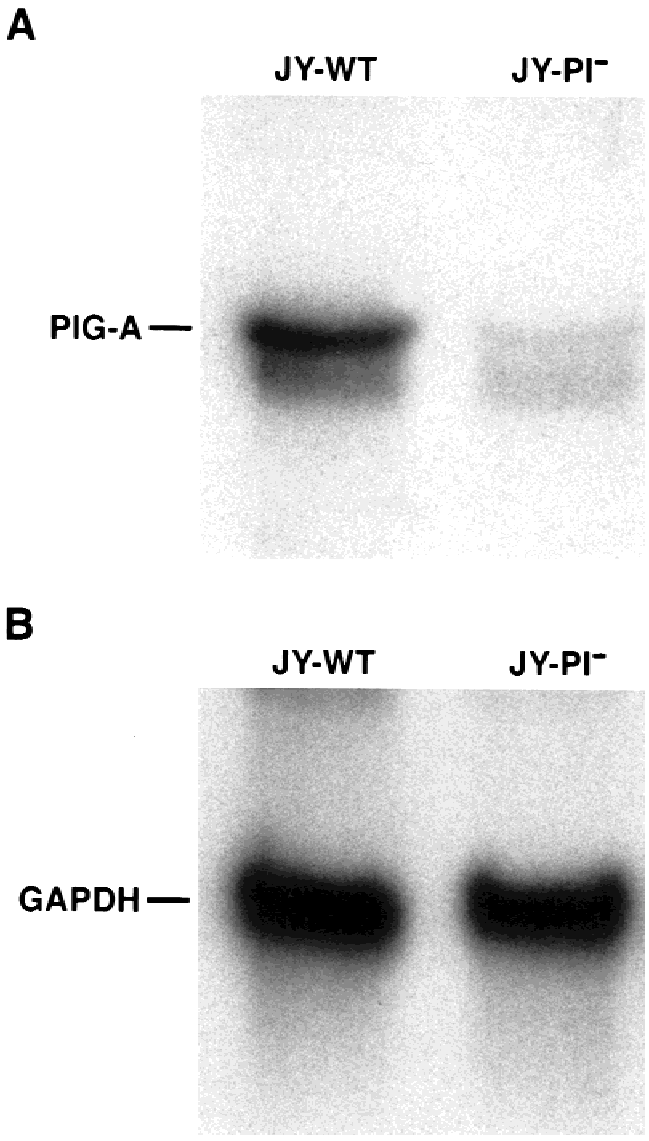

Fig. 2. Northern analysis of *PIG-A* expression. Aliquots of 10 μg of total RNA from JY-WT and JY-PI⁻ cells were analyzed by using a radiolabeled *PIG-A* probe (A). To confirm the integrity of the RNA and to demonstrate that each lane contained a similar amount of RNA, the radioactivity was removed, and the blot was subsequently analyzed by using a radiolabeled GAPDH probe (B). Expression of *PIG-A* by JY-PI⁻ cells was abnormally low.

served as the tester). For DP-1, the ratio of driver:tester was 100:1. Compared to the representational amplicons, some of the bands observed in the lanes containing the DP-1 samples were more discrete, and the intensity of some of the bands appeared different in set A compared to set B (Fig. 3A).

To generate DP-2, portions of DP-1 were digested with *Bgl*II; and after washing to remove the primer/adaptor set that had been cleaved from the PCR products, a different set of adaptor/primers was ligated to each sample in order to create the new tester. The appropriate representational amplicon was again used as driver, and the driver:tester ratio was increased to 800:1. The pattern of the DP-2 samples was similar to that of the DP-1 samples except the bands were more discrete, and the intensity of some of the bands was increased (Fig. 3).

Next, the DP-2 samples were digested with *Bgl*II, washed, and ligated with a different set of adapter/primers. Again the representational amplicons were used as the driver, and the third round of subtractive hybridization was performed by using a driver:tester ratio of 400,000:1. Discrete bands were not observed in the lane containing set A, whereas a prominent band with a mass of approximately 920 bp was present in the lane containing set B (Fig. 3B). These results indicated that JY-WT cells expressed a gene that was not expressed in JY-PI⁻ cells.

Southern and Northern Analysis

An oligonucleotide probe was prepared by ligating DP-3 from set B into a plasmid vector. Southern analysis demonstrated that the radiolabeled probe hybridized to cDNA found in the JY-WT representational amplicon but not in that of the JY-PI⁻ amplicon (Fig. 4). The nucleotide sequence of the DP-3 set B clone was determined and compared to sequence contained in the BLASTN database. Excluding the primer/adaptors, the sequence revealed complete identity with an 870 bp segment of antiquitin cDNA. As anticipated, both the 5′ and 3′ ends of the segment contain *Bgl*II restriction sites.

To confirm that the DP-3 set B sequence was expressed by JY-WT cells but not by JY-PI⁻ cells, Northern analysis was performed. A prominent 1.9 kb band was observed in the lane containing total RNA from the JY-WT cells but not in the lane containing total RNA from JY-PI⁻ cells (Fig. 5). A faint band of 4.4 kb was also noted in the JY-WT lane but not in the JY-PI⁻ lane. Northern analysis using poly (A+) RNA confirmed the observations by using total RNA. These experiments account for the appearance of antiquitin sequence in DP-3 set B because antiquitin mRNA is present in the set B tester (initially prepared from JY-WT cells) and is absent from the set B driver (prepared from JY-PI⁻ cells).

PIG-A encodes a protein that is essential for the normal synthesis of the GPI moiety that serves as the membrane anchor for a variety of proteins. These proteins undergo post-translation modification in which a portion of the COOH-terminus is removed and replaced by the GPI moiety. If antiquitin were a GPI-AP and if mutant *PIG-A* exerted a global influence on the expression or stability of GPI-AP mRNA, the absence of antiquitin mRNA from JY-PI⁻ cells could be a consequence of a generalized process. To investigate this hypothesis, poly(A+) RNA from JY-WT and JY-PI⁻ cells was analyzed by using DAF (CD55) and MIRL (CD59) specific probes [Fig. 6]. As previously reported, three different

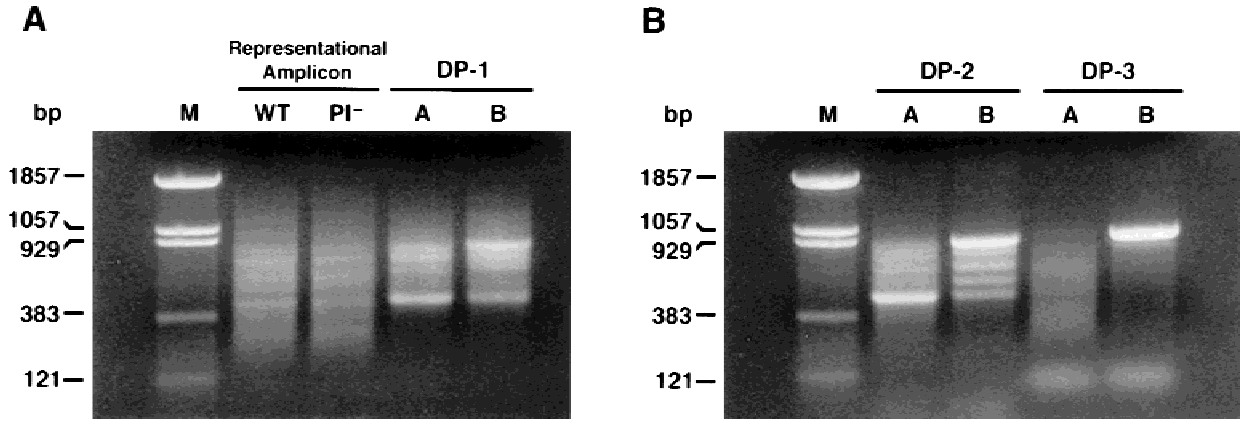

Fig. 3. Comparison of gene expression using RDA. Double stranded cDNA was prepared using poly (A^+) RNA from JY-WT and JY-PI^- cells. Representational amplicons were generated by cleaving the cDNA with *BglII*, ligating the product with a set of adaptor/primers, and amplifying the samples by using PCR. No obvious difference in the appearance of the amplicons was observed when the samples were analyzed by agarose gel electrophoresis and ethidium bromide staining (A). Sequential hybridization/subtraction was performed in both directions. In set A, the JY-WT amplicon was used as the driver and the JY-PI^- amplicon was used as the tester. In set B, the JY-PI^- amplicon was used as the driver and the JY-WT amplicon was used as the tester. To generate difference product 1 (DP-1) a driver:tester ratio of 100:1 was used (A). In the case of DP-2, the driver:tester ratio was 800:1, and the ratio for DP-3 was 400,000:1 (B). The lanes designated M contained the size markers. (Left) the size of the standards in bp is indicated. The presence of a discrete band in the lane containing DP-3 set B suggested that JY-WT cells express a gene that is not expressed by JY-PI^- cells.

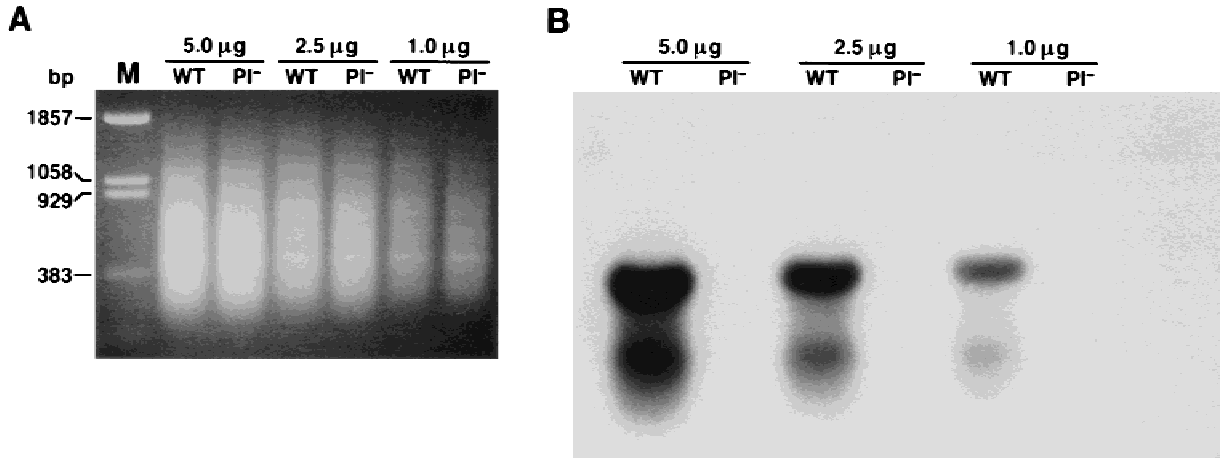

Fig. 4. Southern analysis. Three different concentrations of cDNA were electrophoresed and analyzed by ethidium bromide staining (A) and Southern blot (B) by using a probe generated by cloning DP-3 set B. WT indicates that the cDNA was from the JY-WT representation amplicon and PI^- indicates that the cDNA was from the JY-PI^- amplicon. The lane designated M contained the size markers. (Left) the size of each standard in bp is indicated for the ethidium bromide stained gel. The probe hybridized to cDNA from the JY-WT representation amplicon but not to that from the JY-PI^- amplicon, confirming that DP-3 was a true difference product.

sized transcripts (3.1, 2.7, and 2.0 kb) [19] were observed in the blots hybridized with the DAF probe, and six different sized transcripts (6.0, 2.2, 1.9, 1.2, and 0.6 kb) [20,21] were observed when the MIRL probe was used. The pattern of expression of each gene by the two different phenotypes was quantitatively and qualitatively indistinguishable (Fig. 6). These results suggest that the absence of antiquitin mRNA from JY-PI^- cells is not part of a generalized process affecting expression of all GPI-AP mRNA.

Effects of Transfection of JY-PI^- Cells with *PIG-A* cDNA on Expression of Antiquitin

Transfection experiments were used to determine if the failure of JY-PI^- cells to express antiquitin is a consequence of mutant PIG-A. After 2 weeks, over half of

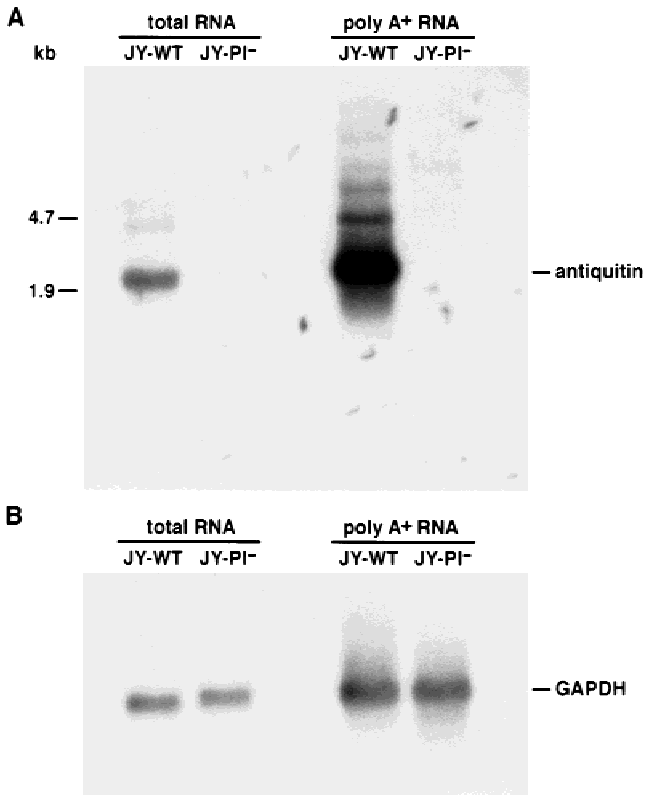

Fig. 5. Northern analysis of antiquitin expression. (A) Total (10 µg/lane) or poly (A^+) (2 µg/lane) RNA isolated from JY-WT or JY-PI^- cells was analyzed by northern blot using an antiquitin specific probe derived from DP-3 set B. (Left) the positions of the 28s and 18s ribosomal RNA bands are indicated, and the size of each in kb is shown. Antiquitin was expressed by the JY-WT cells but not by the JY-PI^- cells. (B) Radioactivity was removed and the blot was subsequently analyzed by using a GAPDH probe. The results confirmed the integrity of the RNA and demonstrated that the paired lanes contained equivalent amounts of RNA.

the JY-PI^- that had been transfected with PIG-A cDNA and grown in hygromycin selection medium expressed normal levels of GPI-AP (Fig. 7, left panel). In contrast, JY-PI^- cells transfected with the control vector containing CAT cDNA (confirmed by a CAT-specific ELISA) failed to express DAF (CD55) and MIRL (CD59). Following an additional 2 weeks growth in medium containing hygromycin, essentially all of the cells transfected with the pEBPIG-A vector were GPI-AP positive, whereas the control cells remained GPI-AP negative (Fig. 7, right panel). These results confirmed that the expression of GPI-AP by the JY-PI^- cells was due to successful transfection with the vector construct containing wild-type *PIG-A* cDNA.

Expression of antiquitin was analyzed by RT-PCR. Successful transfection with pEBPIG-A did not induce antiquitin expression in the JY-PI^- cells (Fig. 8A). The failure of the *PIG-A* transfected cells to express antiquitin was not a consequence of unsuccessful synthesis of cDNA as amplification was observed when actin primers were used in RT-PCR experiments (Fig. 8A). When cDNA was prepared from total RNA derived from antigen nonspecific T cells and from JY-WT cells, antiquitin expression was observed, but neither transfected nor untransfected JY-PI^- cells expressed antiquitin (Fig. 8B). These experiments demonstrated that the absence of expression of antiquitin by JY-PI^- cells was not a technical artifact involving the the RT-PCR procedure. That transfection of JY-PI^- cells with *PIG-A* cDNA did not induce transcription of antiquitin indicates that PIG-A does not regulate expression of the antiquitin gene.

DISCUSSION

The purpose of the experiments reported herein was to determine if the pattern of gene expression differed between cells that were deficient in GPI-AP due to mutant *PIG-A* compared to cells with normal GPI-AP expression. These studies showed a difference in the pattern of gene expression; but at least in this case, the difference was not a direct consequence of mutant *PIG-A.*

Unlike their wild-type counterparts, cells that lacked GPI-AP (Fig. 1) due to mutant *PIG-A* (Fig. 2) failed to express antiquitin (Figs. 3–5). As some GPI-AP function as receptors for soluble ligands [1,30] and appear to be involved in processes associated with cellular activation [31,32], and perhaps apoptosis [33], we hypothesized that deficiency of one or more GPI-AP modified the pattern of gene expression resulting in the absence of antiquitin form the JY-PI^- cells. This hypothesis was challenged by transfecting the JY-PI^- cells with *PIG-A* cDNA. Those experiments showed that while transfection restored expression of GPI-AP (Fig. 7), expression of antiquitin was not restored in the transfected cells (Fig. 8). These results indicate that transcription of antiquitin is not regulated indirectly by *PIG-A* as a consequence of the essential role of *PIG-A* in the normal expression of GPI-AP.

Thus, the basis of the absence of antiquitin RNA from JY-PI^- cells is speculative. Conceivably, the antiquitin structural gene was inactivated by mutation as was the case for *PIG-A.* This explanation seems unlikely, however, because no antiquitin message was detected in the JY-PI^- cells either by Northern analysis of poly(A+) RNA (Fig. 5A) or by PCR (Fig. 8). Unless, like *PIG-A,* the antiquitin gene is located on the X chromosome or unless there is loss of heterozygosity that includes the antiquitin locus, the probability that the mutagenic process would result in the complete absence of message expression seems remote, because the function of two alleles would need to be completely abolished by the mutagenic process. Even in the case of the mutant *PIG-A,*

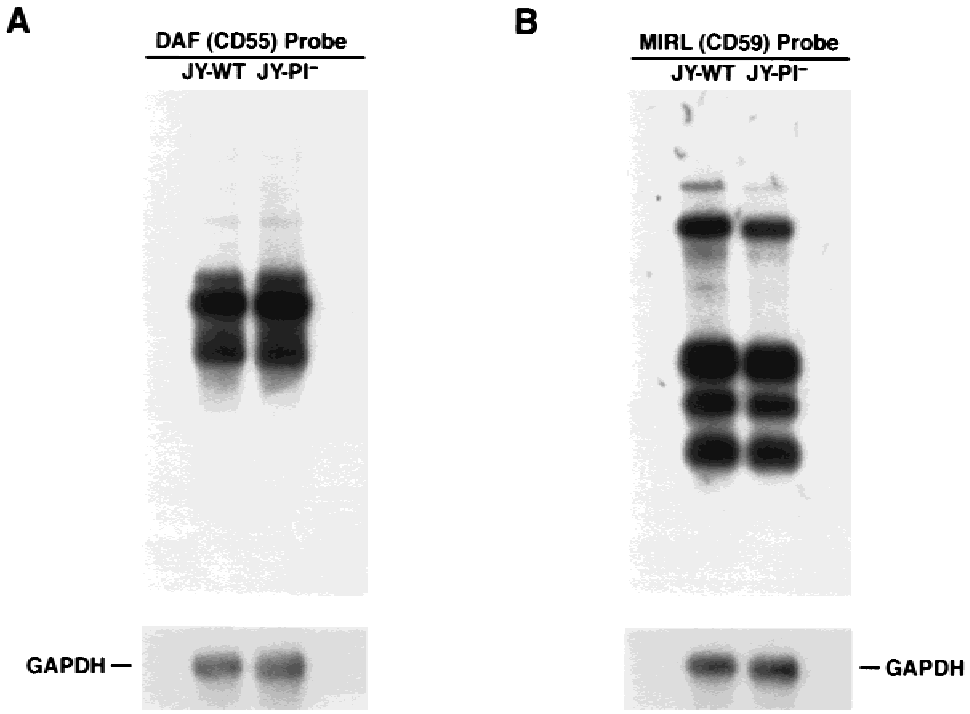

Fig. 6. Northern analysis of DAF (CD55) and MIRL (CD59) expression. Aliquots of 2 μg of poly (A^+) RNA isolated from either JY-WT or JY-PI^- cells were analyzed using probes specific for either DAF (CD55) (A) or MIRL (CD59) (B). No difference in expression of either message was apparent. (Bottom) to confirm that the paired lanes contained equivalent amounts of RNA and that the RNA was not degraded, radioactivity was removed, and the blots were analyzed using a GAPDH probe.

some RNA was detectable by Northern analysis (Fig. 2). Further, no differences in antiquitin gene structure were observed when genomic DNA from JY-WT and JY-PI^- cells was subjected to Southern analysis, and no difference in intensity of the restriction fragments was apparent when DNA from a female was compared with that from a male (data not shown). A genetic basis could still underlie the absence of antiquitin from JY-PI^- cells if the gene encoding a *trans*-acting factor that regulates transcription were inactivated through mutation. The characteristics of the promoter region of antiquitin, however, have not been reported, and currently we have no additional data that address this hypothesis.

As an explanation for the absence of antiquitin expression by the JY-PI^- cells, or conversely, the presence of antiquitin expression by the JY-WT cells, epigenetic factors must also be considered. According to this hypothesis, the pattern of expression of positive or negative regulatory elements that affect antiquitin expression could differ between the two cell lines, and these differences could depend upon stochastic events that influence gene expression.

Heterogeneity is observed in PNH at two different levels. First, among different patients, the extent to which *PIG-A* mutant cells contribute to hematopoiesis is extremely variable with the percentage of abnormal erythrocytes in the peripheral blood ranging from >90% to <5% [5]. Second, in patients with multiple abnormal stem cells, not all of the PIG-A mutant clones contribute equally to hematopoiesis [9,10]. The results of the current studies suggest that some of the heterogeneity in PNH may be due to epigenetic or genetic events that are independent of the *PIG-A* mutations. According to the epigenetic model, among stem cells the pattern of gene expression is not entirely uniform, and these differences produce a pool of cells that is functionally diverse. Thus, the characteristics of a stem cell that undergoes mutation of *PIG-A* would depend upon both the intrinsic properties of the affected stem cell that result from stochastic events and the consequences of the *PIG-A* mutation.

The genetic model of heterogeneity in PNH borrows from observations on the process by which malignancies develop through clonal evolution. According to this hypothesis (modified for PNH), stem cells with mutant *PIG-A* accumulate mutations affecting other genes that in some instances influence the growth or survival characteristics of the affected cells. Thus, clonal evolution could produce the genetic diversity needed to account for the functional heterogeneity of *PIG-A* mutant stem cells [9,10].

The basis of the selective advantage of *PIG-A* mutant stem cells in PNH is speculative, however, in some instances, mutant *PIG-A* appears to contribute directly to clonal expansion. PNH has been shown to arise in association with a number of other clonal myelopathies (e.g., myelodysplastic and myeloproliferative diseases) [5,12]. Under these circumstances, the PIG-A mutation seems to provide an additional proliferative advantage because the affected clone expands sufficiently to allow the clinical manifestations of PNH to become apparent over the background of the other clonal process. These observa-

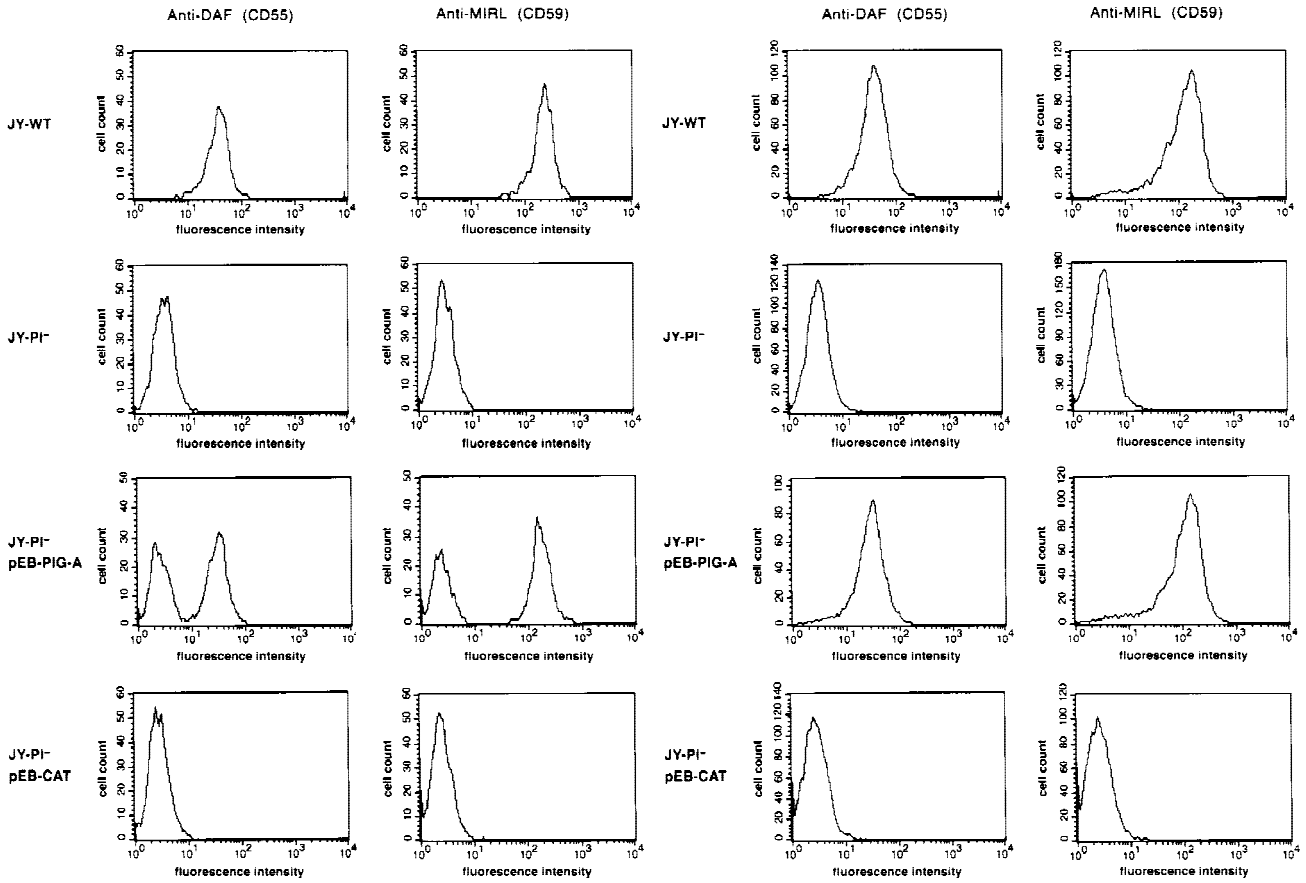

Fig. 7. Effects of transfection on expression of GPI-AP. Expression of GPI-AP by JY-WT, JY-PI⁻, JY-PI⁻ transfected with pEB-PIG-A, or JY-PI⁻ transfected with pEB-CAT was analyzed by flow cytometry by using monoclonal anti-DAF (CD55) or monoclonal anti-MIRL (CD59) as primary antibodies. (Left) Two weeks after transfection; (right) 4 weeks after transfection. Expression of GPI-AP by JY-PI⁻ cells was restored by transfection with *PIG-A* cDNA.

tions suggest that under some circumstances mutant *PIG-A* has oncogenic properties.

Antiquitin was first identified by Lee et al. [34]. The gene was named antiquitin because it was observed to be highly conserved evolutionarily [34,35]. Recent studies by Skvorak et al. [36] have further characterized the gene. Although the function of antiquitin has not been determined experimentally, its deduced primary structure suggests that it is an aldehyde dehydrogenase. Antiquitin shows only 28–34% amino acid identity with other members of the aldehyde dehydrogenase family [37], however, residues and domains that are known to be essential for enzyme function are highly conserved [38,39].

The possibility that antiquitin is involved in the pathophysiology of PNH seems remote. Conceivably, absence of antiquitin expression in *PIG-A* mutant cells has an additive or synergistic effect on cell proliferation, but a mechanistic basis for such an effect is not obvious even though aldehyde dehydrogenases are involved in a number of important cellular process including detoxification of alcohol-derived acetaldehyde, lipid peroxidation, and metabolism of retinoids, corticosteroids, and bigeneic amines [38–40]. More likely, the absence of expression of antiquitin by the JY-PI⁻ cells (or the presence of antiquitin expression by the JY-WT cells) is a characteristic of the particular clone and is unrelated to whether *PIG-A* is mutant or normal. Rather than identifying a new protein that is involved in the pathophysiology of PNH, the studies reported herein have suggested a framework for understanding the mechanism that accounts for the variable contribution that *PIG-A* mutant stem cells make to hematopoiesis in PNH. Our experiments have shown that the pattern of gene expression can be different even between clones that are derived from the same parental line. Depending upon the functional properties of the involved genes, these differences could influence the proliferative or survival properties of the cell. Thus, the heterogeneous contribution that *PIG-A* mutant stem cells make to hematopoiesis in PNH may depend in part upon the pattern of gene expression that is determined by genetic or epigenetic processes that occur independent of the *PIG-A* mutation.

Although antiquitin expression does not appear to be controlled directly or indirectly by *PIG-A*, our studies do

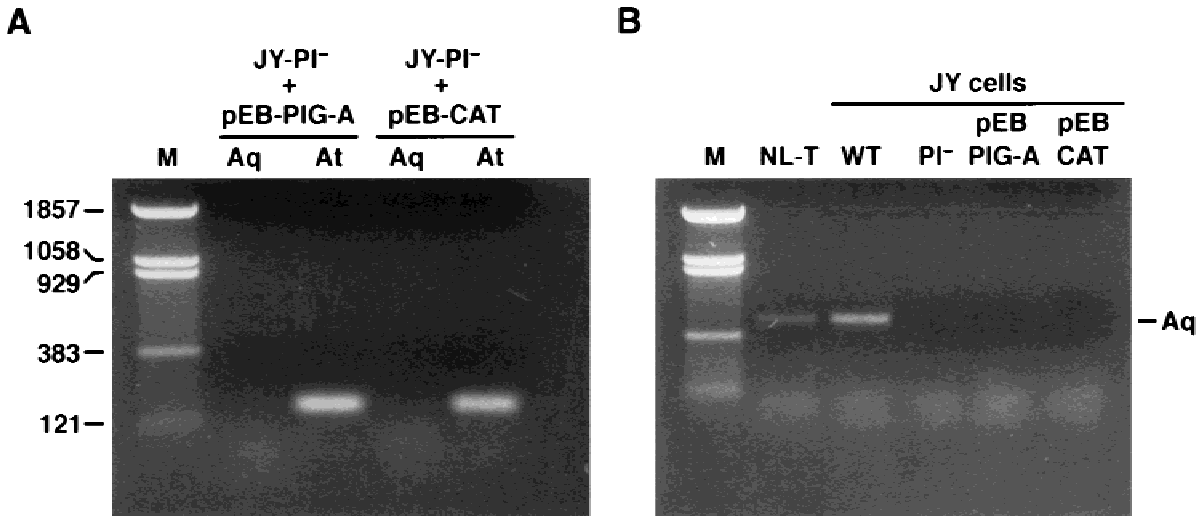

Fig. 8. Analysis of expression of antiquitin by using RT-PCR. (A) Oligo dT primed cDNA was prepared from total RNA isolated from JY-PI⁻ cells transfected with either pEB-PIG-A or pEB-CAT. The cDNA was used as template in PCR experiments by using antiquitin (Aq) or actin (At) primers, and the samples were analyzed by agarose gel electrophoresis and ethidium bromide staining. The lane designated M contained the size markers. (Left) the size of each standard in bp is indicated. The transfected JY-PI⁻ cells expressed actin but not antiquitin. (B) Oligo dT primed cDNA was prepared from total RNA isolated from antigen nonspecific T cells from a normal donor (NL-T), from JY-WT cells (WT), from JY-PI⁻ cells, and from JY-PI⁻ cells transfected with either pEB-PIG-A or pEB-CAT. The cDNA was used as template in PCR experiments by using antiquitin (Aq) primers, and the samples were analyzed by agarose gel electrophoresis and ethidium bromide staining. The lane designated M contained the size markers. Antiquitin was expressed by the normal T cells and the JY-WT cells but not by either the transfected or the untransfected JY-PI⁻ cells.

not exclude the possibility that GPI-AP are involved in gene regulation. For a differentially expressed gene to be identified by RDA, the mRNA must contain two Bgl II restriction sites within a range of ~100 bp to 1,500 bp so that the restriction fragment can be efficiently amplified by PCR. A greater sampling of expressed genes can be achieved by using additional restriction enzymes along with appropriate adaptor/primers [24,25]. Further, the type of cells used for analysis and the conditions under which the cells are grown or manipulated experimentally may affect gene expression. Thus, it is conceivable that under some experimental conditions some cell types that are deficient in GPI-AP may display a pattern of gene expression that is *PIG-A* dependent.

The difference in expression of *PIG-A* by the JY-PI$^+$ cells compared to that by the JY-PI$^-$ cells (Fig. 2) was not identified in the analysis because the portion of the *PIG-A* mRNA that was reverse transcribed by using the oligo dT primers did not contain two *Bgl*II restriction sites (see preceding paragraph). Studies are currently underway using the 4-bp cutter *DPN II* (26) instead of the 6-bp cutter *Bgl*II to generate amplicons. As there are three *DPN II* restriction sites in the coding region, four *PIG-A* amplicons will be generated by using this method. Thus, when cDNA from the JY-PI$^+$ cells is used as the tester, *PIG-A* amplicons will be identified as difference products.

Our studies suggest that genes other than *PIG-A* may contribute to the phenotypic heterogeneity observed in PNH. Nonetheless, it is clear that mutations in *PIG-A* are essential for the development of the disease. Defining the basis of the selective pressure that results in the clonal dominance of *PIG-A* mutant hematopoietic stems cells is essential for understanding the mechanism by which other genes may participate in the pathophysiology of PNH.

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